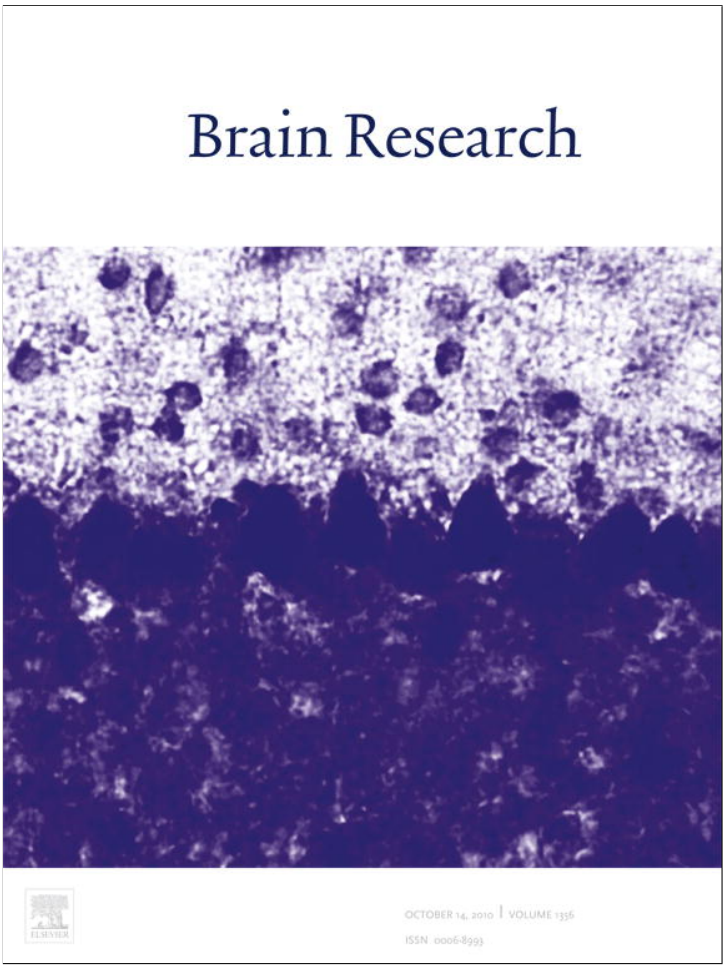

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Research Report

Purkinje cell number decreases in the adult female rat cerebellum following exposure to 900 MHz electromagnetic field

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ABSTRACT

The biological effects of electromagnetic field (EMF) exposure from mobile phones have growing concern among scientists since there are some reports showing increased risk for human health, especially in the use of mobile phones for a long duration. In the presented study, the effects on the number of Purkinje cells in the cerebellum of 16-week (16 weeks) old female rats were investigated following exposure to 900 MHz EMF. Three groups of rats, a control group (CG), sham exposed group (SG) and an electromagnetic field exposed group (EMFG) were used in this study. While EMFG group rats were exposed to 900 MHz EMF (1 h/day for 28 days) in an exposure tube, SG was placed in the exposure tube but not exposed to EMF (1 h/day for 28 days). The specific energy absorption rate (SAR) varied between 0.016 (whole body) and 2 W/kg (locally in the head). The CG was not placed into the exposure tube nor was it exposed to EMF during the study period. At the end of the experiment, all of the female rats were sacrificed and the number of Purkinje cells was estimated using a stereological counting technique. Histopathological evaluations were also done on sections of the cerebellum. Results showed that the total number of Purkinje cells in the cerebellum of the EMFG was significantly lower than those of CG ($p<0.004$) and SG ($p<0.002$). In addition, there was no significant difference at the 0.05 level between the rats' body and brain weights in the EMFG and CG or SG. Therefore, it is suggested that long duration exposure to 900 MHz EMF leads to decreases of Purkinje cell numbers in the female rat cerebellum.

1. Introduction

It has been known that exposure to electromagnetic fields (EMF) has adverse effects on animal tissue and their physiological activities (Dutta et al., 1989; Odaci et al., 2008; Bas et al., 2009a,b; Ragbetli et al., 2010, 2009; Ammani et al., 2010; Maskey et al., 2010). There are also some reports showing increased risk for human health due to long duration of mobile phone

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use (Hardell et al., 1999, 2006, 2007). Therefore, the biological effects of EMF exposure from mobile phones have growing concern among scientists. Today, widespread concerns of mobile phone usage have been raised, since more than 80% of the population use mobile phones in several countries (Feychting et al., 2005).

There are several studies that have emerged which have indicated that EMFs emitted by mobile phones could affect body tissue, systems and their physiologic activities (Mausset et al., 2001; Mausset-Bonnefont et al., 2004; Salford et al., 2003; Koyu et al., 2005; Yildiz et al., 2006; Manikonda et al., 2007). Besides, studies are especially focused on the central nervous system (CNS) since the mobile phone is used in close vicinity to the brain (Lin, 1997; Mausset et al., 2001; Hietanen, 2006; Lahkola et al., 2008). However, the potential adverse effects of EMFs emitted by mobile phones on the human CNS are still controversial (Hietanen, 2006), although its adverse effects on animal brain tissue and their physiologic activities were reported (Salford et al., 2003; Odaci et al., 2008; Bas et al., 2009a,b; Maskey et al., 2010). Some authors have raised the provocative idea that the use of mobile phones could contribute to the formation of brain tumors (Hardell et al., 1999, 2006, 2007) recently, we reported studies that evaluated rat CNS following exposure to 900 MHz (megahertz) EMF during the prenatal or postnatal period. Our results showed that EMF exposure causes a decrease in the number of granule cells in the dentate gyrus (Odaci et al., 2008) and a decrease in the total pyramidal cell number in the cornu ammonis (CA) of the rats (Bas et al., 2009a) following prenatal exposure to 900 MHz EMF. 900 MHz EMF was chosen for the experiment owing to the fact that most mobile phones in Europe generally work at this frequency [in accordance with the Global System for Mobile Communications (GSM)] and the most popular standard for mobile phones in the world (Dubreuil et al., 2003; Koyu et al., 2005; Panagopoulos et al., 2007).

We also found a decrease of the number of pyramidal cells in the CA of the 16-week old female rat hippocampus following postnatal exposure to 900 MHz EMF for 28 days (Bas et al., 2009b). Due to the fact that female humans including adolescents use mobile phones more than male humans and also tend to talk more by mobile phone than males (Söderqvist et al., 2007, 2008), it is claimed that the female gender brain may have more exposure to EMFs than the male. In this study, we want to investigate the effect of EMF on the cerebellum. Since it is known that the cerebellum has substantial connections with the brain cells that point a cognitive role of cerebellum, beside of the control of muscle movement, equilibrium and posture of body. Both neuropsychological and neuroimaging findings supply to this idea (Strick et al., 2009). For that reason we aimed to analyze the effect of postnatal exposure to 900 MHz EMF on the number of Purkinje cells in the cerebellum of the 16-week old female rats using the optical fractionator. This is an unbiased stereological counting technique. Histopathologically, sections of the cerebellum obtained from each group were also examined. To our knowledge, this is the first report focusing on the effects of 900 MHz EMF on the number of Purkinje cells in the adult female rat cerebellum using a stereological technique.

2. Results

2.1. Physical examinations and weight of the female rat

At the 16th week, the physical examination of rats showed no unexpected evidence either in exposure to EMF or SG. Additionally, there was no significant difference between the rats' body and brain weights in the EMFG and CG or SG ($p > 0.05$).

2.2. Purkinje cell numbers in the female rat cerebellum

Purkinje cell numbers of the groups were estimated. The total number of Purkinje cells in the EMFG was significantly lower than those of CG ($p = 0.004$) and SG ($p = 0.002$). However, there was no significant difference between the total number of Purkinje cells of the CG and SG groups ($p > 0.05$) (Fig. 1). The coefficient of variation (CV) and the coefficient of error (CE) of the estimation for the CG, SG, and EMFG are shown in Table 1. It was found that the CE and CV values were within acceptable ranges (Gundersen and Jensen, 1987; West et al., 1991).

2.3. Histopathological observations

At the 16th week, the histological appearance of the female rat cerebellum concerning EMFG, SG and CG is shown in Fig. 2. There was no observable difference found at the sections of the CG and SG groups. On the other hand if the EMFG slides are compared with the SG and CG, a huge amount of Purkinje cell loss is observed.

3. Discussion

The effects of EMF on the female rat cerebellum were investigated in the current study. We estimated the total

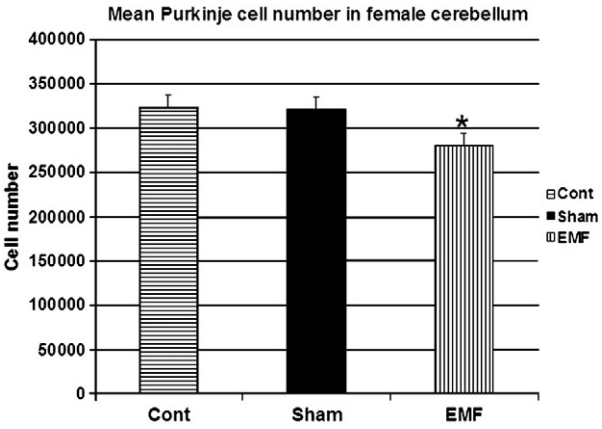

Fig. 1 – The total number of Purkinje cells in the female rats was estimated for the 16-week old EMFG, 16-week old SG and 16-week old CG. The number of Purkinje cells of the 16-week old EMFG female rats was significantly lower than that of the 16-week old SG ($p < 0.002$) and 16-week old CG ($p < 0.004$). However, there was no significant difference between the number of the 16-week old SG and 16-week old CG ($p > 0.05$). 16-week old, 16-week old; CG, control group; SG, sham group; EMFG, electromagnetic field exposed group.

Table 1 – Mean dissector number, section thickness, number of steps, CV and CE of stereological analysis for estimation of total neuron number in the cerebellums of the CG, the SG and the EMFG of rats.

	CG (n=5)	EMFG (n=6)	SG (n=6)
Dissector particle number	209	203	180
Section thickness (μm)	21.20	20.70	20.80
Number of steps for counting	152	148	145
Number of sampled sections	20.60	20.20	20.23
CE	0.07	0.07	0.07
CV	0.03	0.03	0.04

CG, control group; SG, sham group; EMFG, electromagnetic field exposed group.

number of Purkinje cells in the cerebellum of the 16-week old female rats that were exposed to 900 MHz EMF for 28 days. The main reason of using the 16-week old female rats is that they may be comparable with the age of human teenagers. It is known that the CNS of the animal species tested must be compared with that of the same stage of development in the human, regardless of whether it is tested during the fetal, prenatal or postnatal periods (Rodier, 1980; Jacobson, 1991; Odaci et al., 2004; Bas et al., 2009b). For example, it is well known that the neonatal period of CNS development in the rat corresponds to the third trimester of CNS development in humans (Dobbing, 1970; Dobbing and Sands, 1973; Rodier, 1980; Jacobson, 1991). Also, the brain development of the 12–16-week old rats is comparable with the age of human teenagers (Odaci et al., 2004, 2008; Bas et al., 2009b). Our results showed a decrease of the total number of Purkinje cells in the EMFG. This study confirmed the studies of Ragbetli et al. (2010, 2009). This suggests that EMF affects Purkinje cell numbers in the cerebellum of female rats following 900 MHz EMF exposure. Recently, we also reported a decrease of the number of pyramidal cells in the CA of the female rat hippocampus after postnatal exposure to 900 MHz EMF (Bas et al., 2009b).

The effects of exposure to 900 MHz EMF, in our previous studies of hippocampus and in the current study of the cerebellum of 16 week-old female rats were investigated. In both studies, an updated counting technique was used to estimate the total neuron number. Therefore, taking into account both studies, it is suggested that the cerebellum of the 12–16-week old female hippocampus rats are sensitive to exposure of 900 MHz EMFs. These results may be interpreted that the human female teenager's brain may be more sensitive than human male teenager's brain due to the fact that female humans use the mobile phone more than male humans, and they tend to talk more by mobile phone than male humans (Söderqvist et al., 2007, 2008).

EMF has been used in a number of biological and experimental studies. In the last few years, several experimental

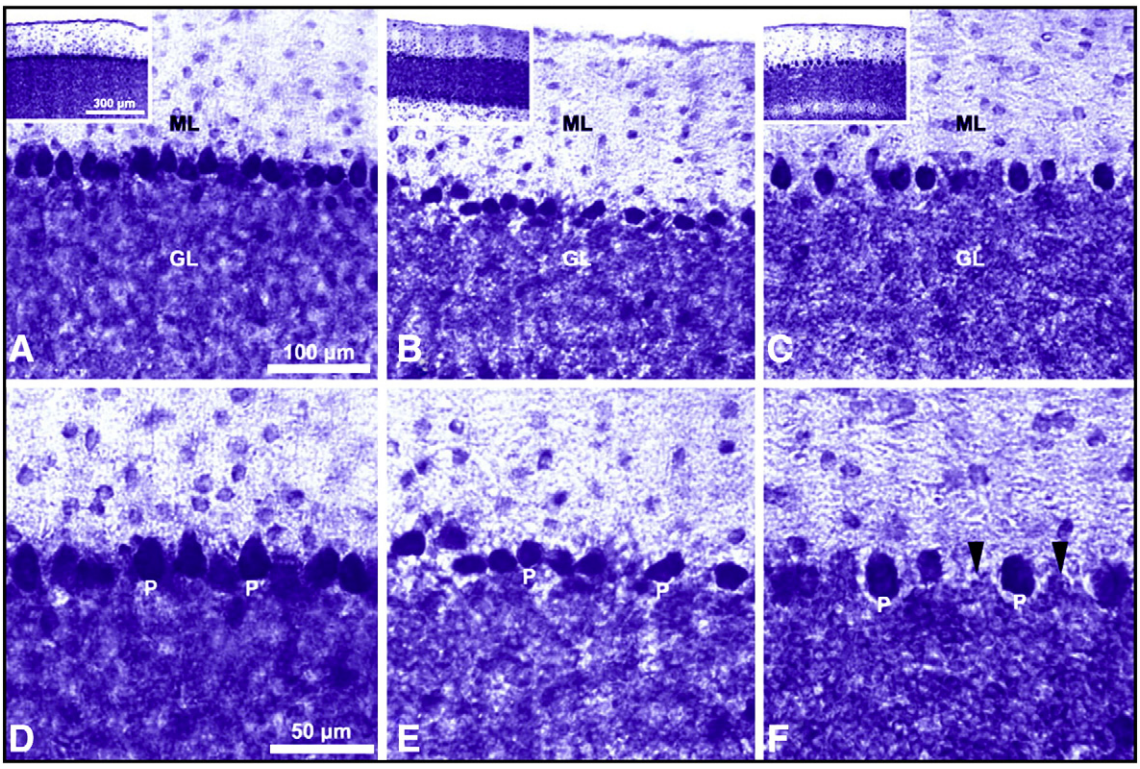

Fig. 2 – The pictures of the groups are shown here. As seen in these pictures substantial cell loss is seen in the EMF group (C, F) in comparison with the CG (A, D) and SG (B, E). It should be noted that such pictures would not be informative since the view of them may be changed by the section plane, tissue shrinkage or swelling, or the area of pictures taken. EMFG, electromagnetic field group; SG, sham group and CG, control group; ML, molecular layer, GL, granular layer; P, Purkinje layer and Purkinje cell. Arrow heads show cell loss in the Purkinje cell layer. Cresyl violet staining.

studies have emerged which have indicated that EMFs emitted by mobile phones could affect brain morphology, its physiologic activities and neurons at the cellular level (Mausset et al., 2001; Mausset-Bonnefont et al., 2004; Salford et al., 2003; Manikonda et al., 2007; Odaci et al., 2008; Bas et al., 2009a,b; Ragbetli et al., 2010, 2009; Maskey et al., 2010). Recently, a study reported that EMF exposure at 835 MHz for 1 month produced almost complete loss of pyramidal cells in the mice CA1 area (Maskey et al., 2010). In this study, calcium binding protein differences were reported showing changes in cellular Ca^{2+} levels. This could have a deleterious effect on normal hippocampal functions concerned with neuronal connectivity and integration (Maskey et al., 2010).

In another study, neuronal damage in the cortex, hippocampus, and basal ganglia in the brains of rats exposed for 2 h to GSM mobile phone EMFs of different strengths was reported (Salford et al., 2003). In this study, when the 12–26-week old rats were used, and scattered and grouped with dark neurons, which were often shrunken and darkly stained, homogenized with loss of discernible internal cell structures were reported histopathologically (Salford et al., 2003). Salford and colleagues also reported that dark neurons increase in the brains of GSM rats after exposure. In addition, the percentage of abnormal neurons was roughly thought to be maximally around 2% (Salford et al., 2003). It was noted that the dark neurons of EMF exposed rats are not apoptotic, as seen with the application of Caspase-3 where apoptosis could not be detected in any of the EMF exposed brains (Salford et al., 2003; Grafström et al., 2008). Recently, Grafström et al. (2008), in a rat model, investigated the effects of repeated exposures under a long period to 900 MHz radiation. In their study, rats were exposed once weekly for a 2 hour period for a total of 55 weeks using different average whole-body SAR values. For this purpose, the animals were exposed to radiation emitted by a GSM-900 test phone. Their histopathological examination showed no significant difference when comparing the brains of the GSM exposed rats to those of the sham exposed rats with regard to the amount of albumin leakage, occurrence of dark neurons, glial reactions, lipofuscin aggregation, and premature or accelerated ageing (Grafström et al., 2008). However, there was an interval of 5–7 weeks between the last EMF exposure and the killing of the animals. This might affect histopathological examinations as stated by Grafström et al. (2008).

Takahashi et al. (2010) evaluated potential adverse effects of long-term whole-body exposure to EMFs simulating those from base stations for cellular phone communication. The pregnant rats were exposed to low, high EMF and no exposure. They found that EMF, 2.14 GHz for 20 h per day during gestation and lactation did not cause adverse effects on the pregnancy or the development of rats. We also noted that there is no a significant difference between groups with regard to the body and brain weight of female rats. These findings were similar to our earlier studies (Odaci et al., 2008; Bas et al., 2009a,b) and also the results of Takahashi et al. (2010). When taken together, it is suggested that EMF exposure does not lead to the loss of body or brain weight following 900 MHz EMF exposure, during prenatal or postnatal period.

Heinrich et al. (in press) conducted a human study to investigate a possible association between radio frequency (RF) EMF and chronic well-being in young persons using personal dosimetry. They collected data on chronic symptoms, socio-demographic characteristics and potential confounders. They found a fatigue as a pronounced chronic symptom in the children and adolescents. Beside this, they did not observe a significant association between measured exposure and chronic symptoms (Heinrich et al., in press). On the other hand, to see the real effect of EMF on the human being is very difficult as stated in the review of van Rongen et al. (2009). To answer some issues of human daily life, like EMF exposure, using voluntary human subjects has great advantages, but on the other hand, often short duration of exposure, small scale of subjects and heterogeneity of people are the big disadvantages of human studies in comparison of inbred animal strains (van Rongen et al., 2009). For these reasons, some points of EMF exposure could not be answered directly from the human studies; naturally animals are used as simulation of human. Relationship between EMF exposure and symptoms like headaches and migraine, and skin itches has not been demonstrated, pointing that psychological factors such as the conscious expectation of effect would play an important role in that condition (van Rongen et al., 2009).

In conclusion, these results show for the first time a cell loss in the female rat cerebellum after exposure to 900 MHz EMF for 28 days. This cell loss can be seen as a result of chronic exposure of the female rat cerebellum Purkinje cell to EMFs. Additionally, our results showed that chronic administration of 900 MHz EMF does not lead to body or brain weight loss following postnatal exposure. This may encourage researchers to evaluate the human cerebellum with regard to EMFs emitted by mobile phones. Besides, additional studies with chronically or varied EMF exposure and duration are required to confirm this and to refine EMF administration in studying the Purkinje cells in the female rat cerebellum.

4. Experimental procedures

4.1. *Animals and study protocol*

Female rats that were obtained from the Experiment Animals Research and Application Center of Afyon Kocatepe University (Afyon, Turkey) were used in this study. They are 16-week old Wistar albino and weighing 270–300 g. Animals housed separately in plastic cages where they rested for two days were maintained on sawdust bedding in an air-conditioned room, 22 ±1 °C, under a 12/12-h light/dark cycle and the humidity ratio was kept at about 40–50%. They were allowed free access to food using a commercially balanced diet (Hasyem Ltd., Isparta, Turkey) and water ad libitum. After the rest period, animals were randomly divided into three equal groups as described previously (Odaci et al., 2008; Bas et al., 2009a,b): the control group (CG) (n=5), the sham exposed group (SG) (n=6), and EMF exposed group (EMFG) (n=6). For the EMFG, rats were exposed to 900 MHz EMF 1 h/day for 28 days in an exposure tube. The rats of SG were placed into the exposure tube without exposure of EMF. The exposure period for the EMFG and SG was daily from 11:00 to 12:00 a.m. No exposure was applied to the rats of CG, and they lived freely in their cages under the normal laboratory conditions without stress (Odaci et al., 2008; Bas et al., 2009a,b). The Animal Ethics Committee of Afyon Kocatepe University approved the

protocol and appropriate measures were taken to minimize pain or discomfort of the animals by our study group.

4.2. Perfusion, fixation and histological procedures

At the 16th week, all the female rats were anaesthetized with 1.25 g/kg of urethane intraperitoneally and perfused intracardially firstly by saline and secondly by neutral formalin. The cerebella were dissected immediately and processed through graded alcohols and xylene, than embedded in paraffin for sectioning. The paraffin block of tissues was cut transversally by a rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany) into serial sections of 30 μm thickness using disposable metal microtome blades (Type N35, Feather Company, Osaka, Japan). Each sampled section through the cerebellum was collected on adhesive coated slides and stained with Cresyl fast violet (Bancroft et al., 1994) for analysis.

4.3. Exposure system design

The exposure system design of the present study is the same as that used in our previous studies (Odaci et al., 2008; Bas et al., 2009a,b). Briefly, the application of electromagnetic field and exposure system used in presented study was described in detail previously (Koyu et al., 2005; Köylü et al., 2006; Yildiz et al., 2006). A specially designated EMF exposure system was used for the exposure of female rats to EMF and stress. The system consisted of a dipole exposure antenna and a round plastic tube cage. An electromagnetic energy generator was manufactured at the Electromagnetic Compatibility Laboratory at Süleyman Demirel University (Koyu et al., 2005; Ozguner et al., 2005; Köylü et al., 2006; Yildiz et al., 2006) for this purpose. It produces 900 MHz continuous modulated EMR (2 W peak output power and 1±04 mW/cm^2 power density).

4.4. Specific energy absorption rate and power density measurements

The specific energy absorption rate (SAR) varied between 0.016 (whole body) and 2 W/kg (locally in the head). In this study, same as our previous experiments (Odaci et al., 2008; Bas et al., 2009a,b) we only obtained the average value in the whole brain but not the SAR value in the area where they looked for morphological changes. Therefore, the brain average SAR value of 2 W/kg showed only the average value in the whole brain. The peak SAR value was obtained by means of model calculations, and the power density measurements were made using an EMF meter (Holaday Industry Inc., Adapazarı, Turkey) (Odaci et al., 2008; Bas et al., 2009a,b).

4.5. Application of electromagnetic field on female rats

The EMF treated rats were positioned in close contact above the dipole antenna and exposed to the EMF (Koyu et al., 2005; Köylü et al., 2006; Ozguner et al., 2005; Yildiz et al., 2006; Odaci et al., 2008; Bas et al., 2009a,b). The distance between them was 1 cm and they were positioned perpendicular to the dipole antenna. The heads of the rats were positioned in the direction of the antenna making the long axis of the rats perpendicular to the long axis of the antenna. The Sham group rats were positioned same as the rats of the EMF group without EMF exposure. The position of rats during EMF exposure was changed daily so that exposure could cover the whole brain (Odaci et al., 2008; Bas et al., 2009a,b).

4.6. Stereological analyses

All stereological analyses were done blind to treatment to obtain unbiased results. Same as the previously published papers (Odaci et al., 2008; Bas et al., 2009a,b) we also used the optical fractionator technique for Purkinje cell counting (West et al., 1991; Ragbetli et al., 2007; Tunc et al., 2007; Odaci et al., 2010). Briefly, the section sampled fraction (ssf) was 1/10 and the first section was selected randomly within the first 10 sections for each cerebellum. About 18 to 23 sections were sampled from each cerebellum in a systematic random manner. The unbiased counting frame size for Purkinje cell estimation was 596 μm^2, the area sampling fraction (asf) was 596 μm^2/90,000 μm^2, and the thickness sampling fraction (tsf) was 10 μm/20.90 μm for the cell count. Although the upper guard zone for tsf was 5 μm, the thickness of the bottom guard zone changed depending on the section thickness.

Stereological analyses were performed at a stereology workstation (Samsun, Turkey). It consists of a CCD digital camera, an image capture card (Flash Point, Integral Technologies, Indianapolis, IN, USA), a personal computer, a computer-controlled motorized specimen stage (Prior Scientific, Cambridge, UK), a microcator (Heidenhein Traunreut, Germany) and a light microscope (Leica, Nubloch, Germany). A software program (CAST-GRID Computer Assisted Stereological Toolbox; Olympus) was used to control, measure and record stereological data and to capture digital images of the sections. This system reproduced microscopy images [obtained through a 100× Leica HCX Plan Apo objective; numerical aperture (NA)=1.40] on the computer monitor at a final magnification of 5140 that allowed accurate recognition and counting for total number of Purkinje cells in the cerebellum.

Details regarding the stereological methods used in the current study have been described previously (Ragbetli et al., 2007; Odaci et al., 2008; Bas et al., 2009a,b; Odaci et al., 2010). The total number of Purkinje cells in the cerebellum was estimated using the optical fractionator technique (West et al., 1991, Ragbetli et al., 2007; Odaci et al., 2008). The Purkinje cells were counted in the cerebellum where the widest profile of the nucleus comes into focus within optical dissector probes systematically randomly spaced throughout the regions of the cerebellum (Ragbetli et al., 2007; Bas et al., 2009a,b). The total number of Purkinje cells in the cerebellum (N) was estimated by means of the following formula:

$$N = \sum Q \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

where ∑Q is the total dissector neuron number, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf is the thickness sampling fraction. Estimation of total number of neurons was calculated from the number of counted neurons and the sampling probability (Gundersen, 1986; Gundersen and Jensen, 1987; West et al., 1991).

By means of the coefficient of error (CE) and the coefficient of variation (CV), the convenience of the sampling scheme in

its precision of the estimates and the convenient number of sampled cells for the estimation of total Purkinje number were checked as previously reported (Gundersen and Jensen, 1987; West and Gundersen, 1990; Thomas et al., 1998). The CE of the sampling schedule for the Purkinje cell was validated (West and Gundersen, 1990; West et al., 1991; Chen et al., 2003). It is also a requirement for a stereological analysis to give of CV within the cerebellum in each group. This valuable data could be used to see whether the number of subjects in each group was sufficient. In the present study, the mean CEs and CVs for the number of Purkinje cells sampled for the groups are given in Table 1. In addition, details of counting procedures and other stereological parameters are also seen in Table 1.

4.7. *Statistical analysis*

All data were expressed as means±standard error mean for each group. Comparisons between the Purkinje cell numbers of the cerebellum were carried out using the Kruskal–Wallis test. It has been known that when the variances in the population are not equal, nonparametric test must be used (Tunc et al., 2007; Odaci et al., 2010). In this study variance was not homogeneous and compared groups were independent. Therefore, we used the Kruskal–Wallis one-way analysis of variance in lieu of parametric single-factor between-subjects analysis of variance. To compare Purkinje cell numbers between the groups, we used one of the nonparametric tests called the Mann–Whitney U test. Mean values were considered to be significantly different at $p<0.05$. All statistical analyses were performed using the SPSS software (Statistical Package for the Social Sciences, version 13.0, SPSS Inc., Chicago, USA).

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